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Paternal RNA contributions in the *Caenorhabditis elegans* zygote

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Abstract

Development of the early embryo is thought to be mainly driven by maternal gene products and post-transcriptional gene regulation. Here, we used metabolic labeling to show that RNA can be transferred by sperm into the oocyte upon fertilization. To identify genes with paternal expression in the embryo, we performed crosses of males and females from divergent *Caenorhabditis elegans* strains. RNA sequencing of mRNAs and small RNAs in the 1-cell hybrid embryo revealed that about one hundred sixty paternal mRNAs are reproducibly expressed in the embryo and that about half of all assayed endogenous siRNAs and piRNAs are also of paternal origin. Together, our results suggest an unexplored paternal contribution to early development.

Keywords embryogenesis; epigenetic inheritance; paternal RNA; transgenerational inheritance

Subject Categories Development & Differentiation; RNA Biology; Systems & Computational Biology

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Introduction

Early embryogenesis mainly relies on maternally provided mRNAs and proteins (Yamamoto *et al*, 2006; Stitzel & Seydoux, 2007; Marcello & Singson, 2010). Sperm is almost exclusively thought to be required for embryogenesis by contributing its haploid genome, inducing calcium oscillations, and delivering the microtubule organizing center to initiate mitotic spindle formation in most organisms (Schatten, 1994; Bornens, 2012). Although somatic cell nuclear transfer experiments for animal cloning (McGrath & Solter, 1983; Gurdon & Melton, 2008) and the successful generation of parthenogenetic mice (Kono *et al*, 2004; Kawahara *et al*, 2007) strongly argue that unfertilized oocytes contain all factors to direct early development, this paradigm is challenged by numerous studies. These describe paternally derived signaling molecules, transcription factors and histone variants in zygotes with potential function in development (Wood *et al*, 1980; Hill *et al*, 1989; Shalgi *et al*, 1994; Sette *et al*, 1997; Ooi *et al*, 2006; Yoon *et al*, 2008). It is widely accepted that mature sperm from worms to humans contains a variety of RNA species (Ward *et al*, 1981; Ostermeier *et al*, 2002; Rassoulzadegan *et al*, 2007a,b; Han *et al*, 2009; Liu *et al*, 2012; Conine *et al*, 2013; Sendler *et al*, 2013). However, *in vivo* delivery of spermatozoal RNA into the oocyte and the role of sperm-derived RNAs remain controversial (Miller *et al*, 2005; Lalancette *et al*, 2008; Barroso *et al*, 2009) in part because studies that tried to show direct inheritance of RNA were conducted using non-physiological *in vitro* fertilization assays (Ostermeier *et al*, 2004). Especially in *Caenorhabditis elegans*, inheritance of small RNAs as a potential mode of epigenetic inheritance is an emerging topic. For instance, studies have shown that an exogenous RNAi trigger in males can be inherited to the offspring, while the mode of inheritance most likely functions through small RNAs in sperm (Grishok *et al*, 2001; Alcazar *et al*, 2008; Rechavi *et al*, 2011; Buckley *et al*, 2012; Gu *et al*, 2012). In any case, although studies have recently postulated transfer of small non-coding RNAs by comparing their expression in oocytes, sperm and early embryos (Liu *et al*, 2012; Gapp *et al*, 2014), to our knowledge, there has been no direct test under physiological conditions if sperm can deliver RNAs into the oocyte and if this RNA could be functionally important.

In this study, we used the nematode *C. elegans* to address these questions. We first metabolically labeled the RNA of male worms. After crossing with unlabeled females, we found that about 10% of the embryonic RNA is labeled. We then show that RNA–protein cross-linking of this labeled RNA in the embryo induces embryonic lethality. These results provide evidence that paternal RNA is transferred into the embryo and that this RNA could be functionally important. To reveal the identity of paternal RNA molecules, we performed a cross of males and females from two divergent *C. elegans* strains because we reasoned that sequencing of embryonic RNA and SNP analysis should then identify and quantify maternal and paternal transcripts. These sequencing experiments were carried out in purified hybrid 1-cell embryos and comprised small RNAs and mRNAs. Our data show that paternal RNA reproducibly contributes to the expression of about one hundred sixty mRNAs in the 1-cell embryo. Moreover, about half of all endogenous siRNAs and piRNAs for which we could by SNP analysis assign maternal or paternal genotypes had detectable paternal expression in the 1 cell embryo. For miRNAs, we did not have enough SNPs to delineate

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maternal or paternal transcripts. We therefore complemented our data by small RNA sequencing in wild-type oocytes, sperm, and 1-cell embryos. These data showed that 79 miRNAs are up-regulated in the fertilized embryo compared to oocytes and that this set of miRNAs is particularly highly expressed in sperm, arguing that miRNAs in the embryo could also be in part of paternal origin.

Results

10% of embryonic RNA is of paternal origin

Since it is unknown whether and to what extend RNA can be transferred by sperm into the oocyte, we tested inheritance of metabolically labeled RNA. To this end, worms were grown for one generation in the presence of tritium-labeled uridine (^{3}H-uridine) or thio-labeled guanosine (6-thio-guanosine, 6SG). We had previously shown that nucleoside analogues incorporate into the RNA of the nematode without apparent toxicity (Jungkamp *et al*, 2011). RNA-labeled males were then crossed with unlabeled females, or vice versa, and the RNA of the offspring F1 embryos was subsequently probed for the RNA label (Fig 1A). When crossing labeled males to unlabeled females, with both independent methods, we detected roughly 10% of labeled RNA in offspring embryos (Fig 1B). This suggests that approximately 10% of embryonic total RNA is of paternal origin.

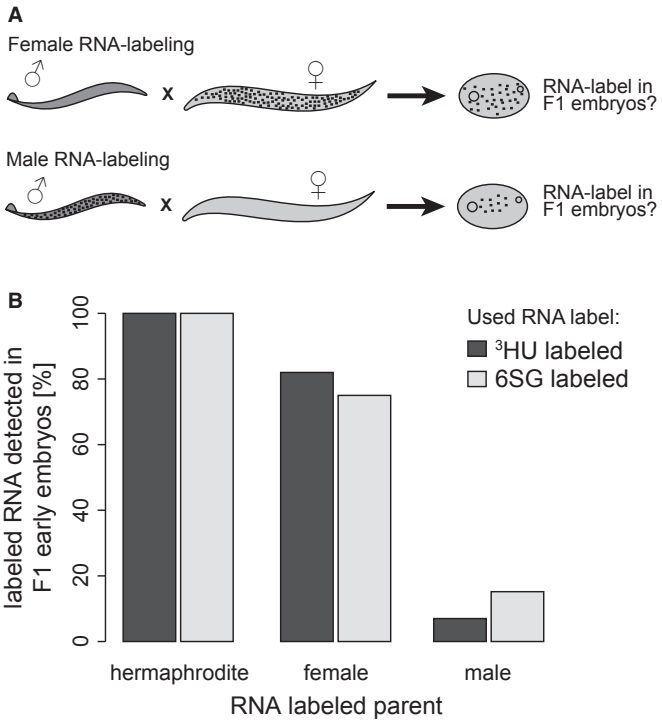

Figure 1. Inheritance of RNA.

A Schematic overview of the assay to measure inherited RNA by uniparental RNA labeling and crossing. To assay whether sperm transfers RNA into the oocyte, RNA was metabolically labeled (tritium-labeled uridine, ^{3}H-U, and 6-thio-guanosine, 6SG) in male worms and crossed to non-labeled feminized hermaphrodites (*fem-1*(*hc17*ts) mutant) and vice versa. RNA isolated from offspring F1 early embryos was then assayed for the RNA label used.

B Depicted is the measured ^{3}H-U (dark gray bars) and 6SG (light gray bars) label in total RNA of F1 early embryos from labeled hermaphrodites, and when only one parent was labeled. ^{3}H-U incorporation into RNA was measured by scintillation counting of isolated total RNA, 6SG by dot blotting total RNA and probing for thio group. When mothers were labeled, around 80% of labeled RNA (compared to when hermaphrodites were labeled) was retrieved in early embryos. When fathers were labeled, around 10% of labeled RNA was retrieved in early embryos.

Cross-linking of paternal RNAs in early embryos causes embryonic lethality

We next asked whether interfering with the complement of RNA transferred by sperm into the oocyte has any effect on embryogenesis. For this, we further exploited the characteristics of thio-labeled nucleoside analogues, which upon irradiation with low-energy UV light have the ability to form intermolecular RNA–RNA and RNA–protein cross-links (Favre *et al*, 1986). We have previously applied this to *C. elegans* and shown that sufficiently well labeled worms die if irradiated at a wavelength that specifically induces these cross-links (Jungkamp *et al*, 2011). This reflects the functional importance of RNA molecules in the worm, and therefore, this method can provide evidence for phenotypes induced by loss of RNA function. We could thus test the hypothesis that interactions between paternal RNAs and proteins are functionally important by labeling the RNA from males and crossing them with unlabeled females and vice versa, followed by irradiation of embryos with UV light inducing specific cross-links (Fig 2A).

We first confirmed that neither RNA labeling nor a UV light pulse alone caused embryonic lethality: F1 embryos from worms grown for one generation in the nucleoside analogues, as well as F1 embryos from worms grown without label but irradiated with UV light, developed normally (Fig 2B). In contrast, embryogenesis was specifically arrested in F1 embryos from hermaphrodites grown in the label and irradiated with UV light (Fig 2B). When performing uniparental RNA labeling (Fig 2C), most embryos from labeled mothers and non-labeled fathers arrest as expected at any cell stage throughout embryogenesis at which RNA was cross-linked (Fig 2C). However, most embryos (~95%, Fig 2C) from labeled fathers and unlabeled mothers also died when cross-linking was induced very early in embryogenesis (1- to 8-cell stage) while approximately 40% of the embryos could develop without obvious defects when cross-linking was triggered around the 55 to approximately 95-cell stage (Fig 2C). These data suggest that RNA transferred by sperm can be functionally important during early embryogenesis.

Identification of paternal and maternal RNAs in the zygote

To identify maternal and paternal RNAs in the zygote, we profiled mRNA and small RNA expression in 1-cell stage embryos generated by a cross of two divergent *C. elegans* strains and performed SNPs analysis (Fig 3A). More specifically, we crossed males from a Hawaiian *C. elegans* strain (CB4856) to a feminized strain with Bristol background (carrying a temperature sensitive *fem-1* allele, hereafter referred to as reference strain) and sequenced the RNAs of the hybrid offspring 1-cell embryos (Fig 3A and Materials and Methods). Fixed 1-cell embryos were automatically collected by a cell-sorting-based method that we established previously (Stoeckius

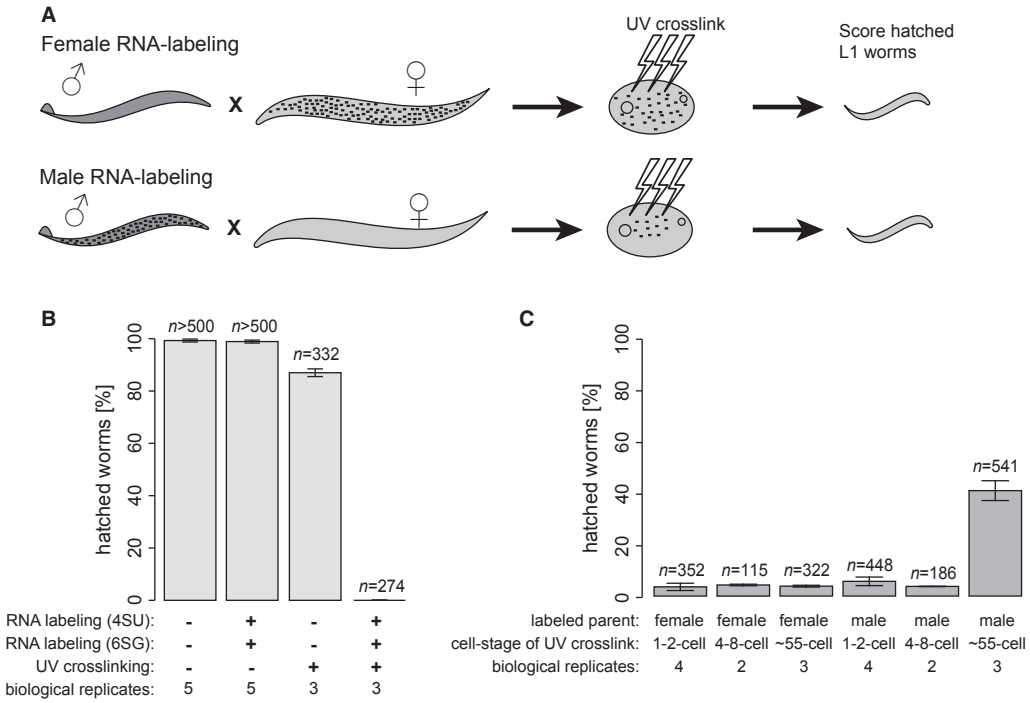

Figure 2. Cross-linking of inherited RNA.

A Schematic overview of the assay to test for functionality of transferred RNAs. We performed uniparental RNA labeling with 4SU and 6SG followed by irradiating the embryos at a wavelength that specifically induces cross-links with the nucleoside analogues.

B Barplots depicting percentages of hatched L1 larvae upon RNA labeling without UV cross-linking, UV cross-linking without RNA labeling, and both, RNA labeling and UV cross-linking. F1 embryos of parental worms that were labeled with 4SU and 6SG develop without obvious defects to feeding larvae, but die upon irradiation with 3 J of 365 nm UV light that specifically photoactivates 4SU and 6SG.

C Barplots depicting percentages of hatched L1 larvae after irradiation with 3 J of 365 nm UV light at different cell stages and different labeled parents. Around 95% of F1 embryos from 4SU- and 6SG-labeled mothers and non-labeled fathers die upon UV irradiation at all assayed cell stages (1-cell stage, 4–8-cell stage and > 55-cell stage). In contrast, while also around 95% of F1 embryos from labeled fathers and non-labeled mothers die upon UV irradiation early in embryogenesis (1–2-cell and 4–8-cell stage), only 60% of embryos die if irradiated around the 55–95-cell stage.

et al, 2009). The Hawaiian strain has roughly one mutation (hereafter referred to as single nucleotide polymorphism (SNP)) every 1,000 nucleotides in the genome compared to strains with Bristol background. Given that 1-cell embryos are transcriptionally silent (Seydoux & Dunn, 1997; Baugh, 2003; Guven-Ozkan *et al*, 2008), a Hawaiian-specific nucleotide in the mRNA of the 1-cell embryo, if matched to the Hawaiian DNA genotype at this position, can serve as a proxy for the paternal contribution in the zygote (Fig 3A).

Because not every transcript has a Hawaiian-specific mutation, this method allowed us to estimate maternal and paternal contribution for roughly 46% of all mRNAs that are expressed in the 1-cell embryo. We could reproducibly detect a paternal contribution for 164 transcripts with 192 SNPs in total in the 1-cell embryo in two independent biological replicates of crosses (Fig 3B; Supplementary Table S1). We observed a strong paternal contribution (stronger than the maternal contribution) for 34 transcripts while 6 transcripts were of predominantly paternal origin (with < 10% maternal contribution) (Supplementary Table S1). To validate our SNP calling and our quantification of the paternal contribution, we tested four candidate SNPs by Sanger sequencing cDNA from independently picked 1-cell embryos, originating from the reference strain, the Hawaiian strain and a cross between the two (Fig 3C). We could confirm the predicted Hawaiian-specific nucleotides and the annotated base in the reference strain. We also observed the predicted SNP in the embryo and a paternal contribution in strength comparable to our sequencing results (Fig 3C).

About half of the paternal contribution is reproduced in our two independent experiments (Fig 3B). Could the non-reproducibility of the other half be due to low read coverage or expression? This seems not to be the case since the fraction of paternal SNPs in transcripts that were reproduced only weakly depends on expression levels. We therefore think that most of the observed variability is due to slightly different biological conditions in the two replicates and hypothesized that part of the paternal contribution might be sensitive to external conditions. Consistently, GO term functional enrichment analysis revealed that genes associated with regulation of growth rate ($P < 1.6e\text{-}7$) and cellular component size ($P < 2.3e\text{-}5$) were highly statistically significantly over-represented among genes with reproducible paternal component, while stress response was the most highly significantly enriched functional category for genes with non-reproducible paternal component ($P < 4e\text{-}10$) and not statistically significantly over-represented among the reproducible genes.

To further investigate the potential paternal origin of these transcripts, we profiled mRNA expression in sperm and spermatocytes

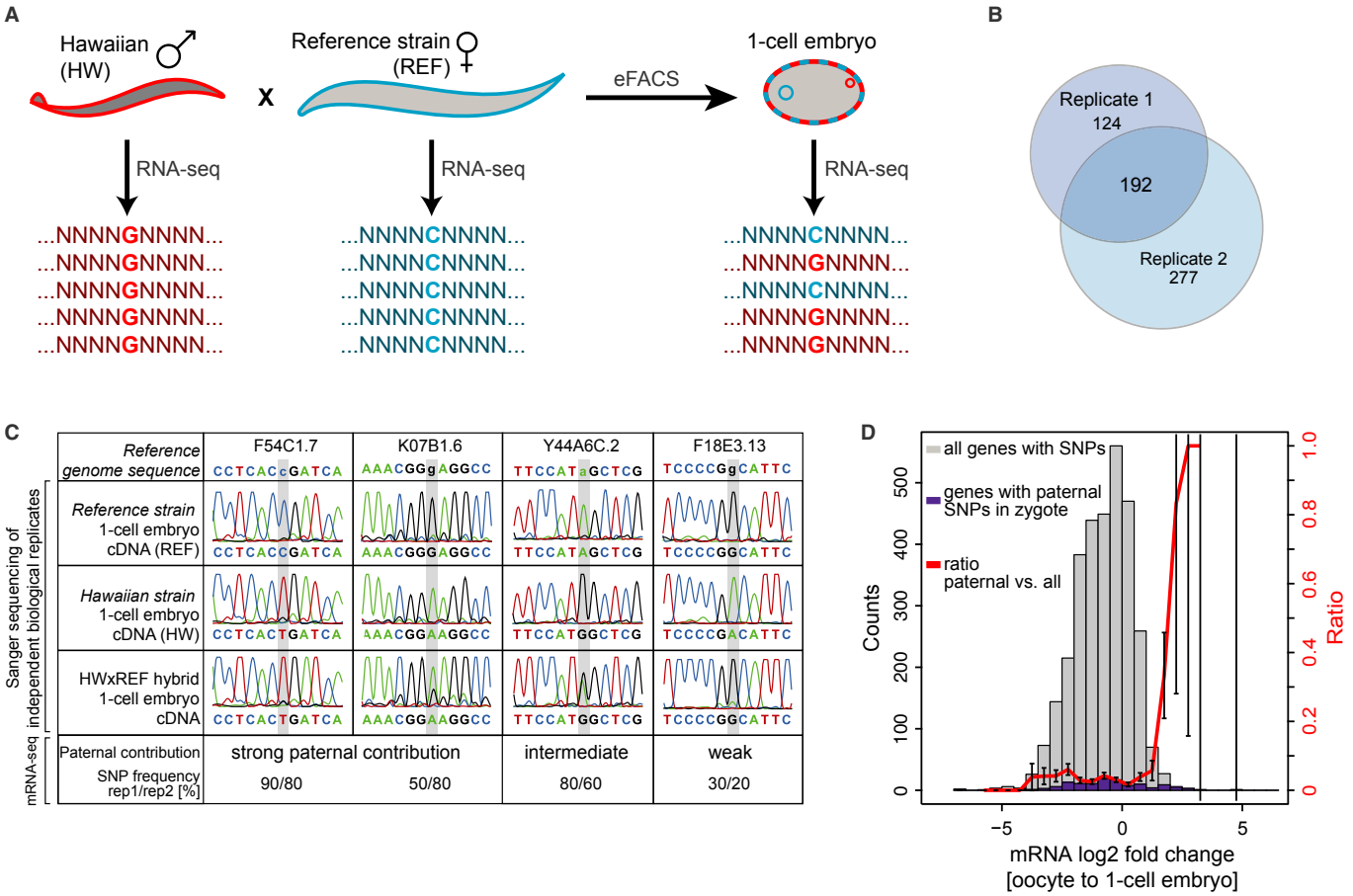

Figure 3. Pinpointing paternal mRNA contribution with SNPs.

A Schematic overview of the crossing experiment performed to pinpoint paternally derived mRNAs in the zygote by Hawaiian-specific mutations. In a transcriptionally silent 1-cell stage embryo, paternally and maternally derived transcripts can be identified by mRNA sequencing assaying mutations specific for the parental strain. HW, Hawaiian strain; REF, reference strain (*fem-1*(*hc17*ts), OMA-1::GFP strain).

B Reproducibility of the number of paternal SNPs in the 1-cell embryos in two independent biological replicate crosses of the Hawaiian strain and our reference strain.

C Sanger sequencing of cDNA from handpicked 1-cell embryos of the Hawaiian strain, the reference strain and a hybrid cross between the two strains for independent biological replicates. Gray boxes highlight the nucleotides with mRNA-seq-based SNP annotation. Electropherogram derived from Sanger sequencing indicates the abundance of a profiled nucleotide. Red = thymine, blue = cytosine, black = guanine, green = adenine. The lower panel lists the paternal contribution as computed from sequencing of two biological replicates (percentage of transcripts with paternal SNP). The ratio of paternal versus maternal nucleotide frequency in the electropherogram at the marked position is in good correspondence with our sequencing results, given the variability of the contribution across biological replicates.

D Histogram of mRNA fold changes between oocytes and 1-cell embryos of all transcripts with identified mutations in the Hawaiian strain (gray) and for the subset of transcripts with corresponding SNPs in 1-cell embryos (purple). The ratio of the two histograms (red line) is increasing toward pronounced positive mRNA fold changes to over 80% of the most highly up-regulated transcripts having a paternal contribution. Error bars were estimated based on random counting statistics.

isolated from males (Supplementary Fig S1A). Genes with paternal contribution in the 1-cell embryo were also expressed in spermatocytes and sperm. However, these genes were not particularly highly expressed in sperm (Supplementary Fig S2A). Moreover, we do not observe paternal transcripts coding for highly expressed sperm genes, such as major sperm proteins (Supplementary Fig S1) in the embryo. These data argue for a specific selection of mRNAs that are transferred into the oocyte upon fertilization.

Genes with paternal contribution in the zygote are mostly uncharacterized genes and do not fall in any specific functional category. However, compared to the set of all expressed genes containing at least a single SNP that allows discrimination of parental contributions, we measured enrichment of RNAi phenotypes in general ($P < 5e\text{-}3$, Fisher's exact test) and specifically for embryonic lethal ($P < 7.9e\text{-}3$, Fisher's exact test) and maternal sterile ($P < 5e\text{-}3$, Fisher's exact test) phenotypes. These phenotypes could potentially explain our observed embryonic lethal RNA-cross-linking phenotypes (Fig 2C).

Transcripts with paternal SNPs in the zygote are also up-regulated at the oocyte-to-embryo transition

In *C. elegans*, RNA polymerase II is transcriptionally silent in the embryo until the 3–4-cell embryo stage (Seydoux & Dunn, 1997; Baugh, 2003; Guven-Ozkan *et al*, 2008). Thus, we reasoned that if sperm transports mRNAs into the oocyte, we would expect these transcripts to be up-regulated in the 1-cell embryo compared to the unfertilized oocyte. We thus closely examined our mRNA sequencing

data of a parallel study (Stoeckius *et al*, 2014), where we profiled mRNA expression by sequencing of polyadenylated transcripts (mRNA-seq) in oocytes and 1-cell stage embryos in N2 worms, that is, in different genetic backgrounds compared to the crossing experiment. The data suggest that 193 mRNAs are significantly up-regulated more than two-fold upon fertilization ($P < 0.05$). 14 out of 16 mRNAs that are up-regulated more than four-fold after fertilization contain paternal SNPs in our Hawaiian crossing experiment ($P \sim 0$, Fisher's exact test) arguing for a paternal contribution (Fig 3D). We next tested whether we could quantitatively validate the paternal mRNA contribution by subtracting the amount of paternal transcripts as computed from the SNP frequency from the transcript level in the 1-cell embryo to achieve an improved regression to the oocyte mRNA expression levels. Interestingly, in comparison to the unmodified embryonic transcript levels, we observed a significant increase in correlation to oocyte mRNA levels after *in silico* subtraction of the paternal contribution from 1-cell mRNA expression ($R^2 = 0.78$ versus $R^2 = 0.66$, Supplementary Fig S2B). The significance of this observation was reflected by a clear shift of the distribution of residuals ($P <$ 1e-9, Wilcoxon's rank sum test, Supplementary Fig S2B).

Moreover, in another independent approach, we directly compared mRNA expression in oocytes and 1-cell stage embryos to sperm (and spermatocytes). For the majority (> 80%) of mRNAs that are highly up-regulated in the 1-cell embryo, we observe higher expression in sperm (and spermatocytes) compared to oocytes, which further suggests that the up-regulated transcripts are of paternal origin (Supplementary Fig S2C).

Transcripts that are up-regulated (> 2-fold) in the zygote are apparently very lowly expressed in oocytes (on average ~0.3 RPKM) and exhibit still very low, but significantly higher expression in 1-cell stage embryos (on average ~ 3.2 RPKM) compared to oocytes, thus leading to a strong positive fold change. For comparison, the 2,000 most highly expressed transcripts in the 1-cell stage have RPKM values ranging from 100 to 24,000 with a mean of around 300 RPKM (Supplementary Table S2). The low expression of these genes in the 1-cell embryo would be consistent with this hypothesis, given the small volume of sperm, limiting its capacity to deliver mRNAs into the zygote.

Evidence for sperm deposited small RNA in the 1-cell embryo

Finally, we asked whether we could find an indication for sperm-derived small RNAs in the zygote using the same approach as for mRNAs. Due to the length of small non-coding RNAs, the probability of bearing Hawaiian-specific nucleotides is smaller than for mRNAs. In fact, mature miRNAs that are expressed in our samples did not contain Hawaiian-specific SNPs, thus making it impossible to delineate paternal and maternal contributions as we did for mRNAs. However, a fraction of the large number of expressed endogenous siRNAs (22G- and 26G-RNAs) and piRNAs (21U-RNAs) in the zygote had Hawaiian-specific SNPs. We found that half of all expressed endo-siRNAs (53%; 803 out of 1,514; Supplementary Fig S3F; Supplementary Table S3) with a SNP had a paternal-specific nucleotide in the 1-cell embryo. Similarly, we observe a paternal contribution for half of all 21U-RNAs with a SNP (51%; 22 out of 43; Supplementary Fig S3E; Supplementary Table S3). Moreover, we could demonstrate that *in silico* subtraction of the SNP frequency-derived paternal contribution from 22G-RNA expression in the embryo improves the correlation with expression in the oocyte (Supplementary Fig S3G). Thus, the paternal contribution partially explains expression changes of 22G-RNAs after fertilization.

To further investigate potential inheritance of small non-coding RNAs, we sequenced small RNAs in oocytes, 1-cell embryos and sperm (Materials and Methods). Small RNA sequencing revealed that the overall small RNA composition was comparable between oocytes and 1-cell embryos, with a slight increase in piRNAs (21U-RNAs) in the 1-cell embryo (Fig 4A and B; Supplementary Table S4). While overall 21U-RNA content in sperm and spermatocytes was also comparable to the 1-cell embryo, mature miRNAs were overrepresented in sperm and spermatocytes (Fig 4A and B). Interestingly, among all small RNA classes, mature miRNAs exhibited the most dynamic expression changes between oocytes and 1-cell embryos, with 35 miRNAs that had a four-fold higher expression in the zygote (Supplementary Fig S3A). After successfully validating miRNA expression changes for ten randomly selected miRNAs by RT-qPCRs normalized to an exogenous spike (Supplementary Fig S3B; Materials and Methods), we hypothesized that the up-regulated miRNAs could potentially be transferred by sperm into the oocyte. We thus asked whether miRNAs that are more highly expressed in 1-cell embryos compared to oocytes are also more highly expressed in sperm. Indeed, 36 (77%) of the 47 miRNAs that were at least twofold up-regulated in the 1-cell embryo versus oocyte were also at least twofold up-regulated in sperm versus oocyte (Fig 4C; Supplementary Fig S3C and D).

In summary, we provide evidence for a paternal contribution of endo-siRNAs and 21U-RNAs in the zygote. Moreover, the data argue that miRNAs in the embryo are also in part of paternal origin.

Discussion

A number of recent studies have provided evidence that sperm from plant to human contains a variety of RNAs that could potentially be transferred into the oocyte at fertilization (Lalancette *et al*, 2008; Barroso *et al*, 2009; Nodine and Bartel, 2012). In *C. elegans*, ultrastructural studies in the early 1980s have already described a yet to be characterized perinuclear halo of RNA in mature sperm (Ward *et al*, 1981), and other studies have recently shown that *C. elegans* sperm contains endogenous siRNAs (Han *et al*, 2009; Conine *et al*, 2013) and that an exogenous RNAi trigger can be inherited most likely by small RNAs to subsequent generations (Grishok *et al*, 2001; Alcazar *et al*, 2008; Rechavi *et al*, 2011; Buckley *et al*, 2012; Gu *et al*, 2012).

In two independent experiments, we reproducibly observed hundreds of paternally derived mRNAs in the zygote. These experiments indicate that the paternally contributed mRNAs are selectively transferred and are not a random spillover effect. We note that these numbers are most likely underestimates since the Hawaiian strain used in the crossing experiments has only limited coverage with Hawaiian-specific genomic nucleotides. According to our estimates, roughly 60% of paternal contributions would not be discoverable by our approach. We also note that the detected paternally derived RNAs could be in principle transcribed from the paternal DNA in the 1-cell embryo or a contamination of < 2% older embryos in our 1-cell embryo

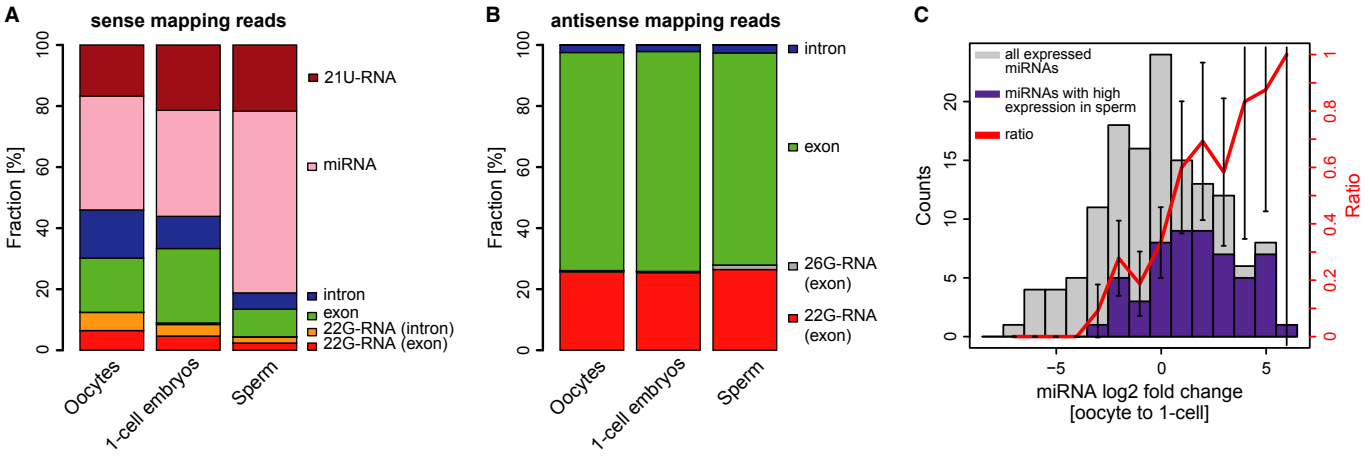

Figure 4. Inheritance of small non-coding RNAs.

A The plot depicts the fraction of sequencing reads that mapped sense to known transcripts and small non-coding RNAs in oocytes, 1-cell embryos and sperm.

B The plot depicts the fraction of sequencing reads that mapped antisense to known transcripts and have a length of 22–26 nt. Most of these reads are most likely endogenous siRNAs as they are 22 nucleotides in length.

C Histogram of log2-fold changes between oocytes and 1-cell embryos for mature miRNAs (gray). A histogram for the subset of miRNAs that were at least twofold up-regulated in sperm versus oocytes is shown in purple. The ratio of the two histograms (red line) increases toward pronounced positive miRNA fold changes, suggesting that the fold change of more than 80% of the most highly up-regulated miRNAs in the 1-cell embryo could be explained by a contribution from sperm. Error bars were estimated based on random counting statistics.

sample obtained by eFACS. However, we believe that this scenario is unlikely because (i) it has been shown that RNAPII is silent until the 3–4-cell stage (Seydoux & Dunn, 1997; Baugh, 2003; Guven-Ozkan *et al*, 2008) and (ii) we do not observe expression of genes that are known to be highly expressed after zygotic genome activation in early embryos (e.g., *vet-4*, *end-1*, *med-1*, *end-3*; Supplementary Fig S1A) or expressed in older embryos (e.g., *myo-3*, *ges-1*, *unc-89*). We also provide evidence that a large proportion of miRNAs, piRNAs and endogenous siRNAs (21U- and 22G-RNAs) are contributed from males. We caution that we cannot exclude the possibility that some of the endo-siRNAs that we suggest are sperm derived are generated in the embryos from sperm-transferred primary transcripts. Nevertheless, our metabolic RNA-labeling experiments (Fig 1) provide evidence that substantial amounts of paternal RNA (~10% of the RNA in early embryos) are transferred into the 1-cell embryo. Moreover, in an independently generated dataset of mRNA expression between oocytes and 1-cell stage embryos, we provide evidence that a subset of mRNAs is up-regulated after fertilization (Stoeckius *et al*, 2014). Over 80% of these strongly up-regulated mRNAs can be explained by paternal SNPs in our crossing experiment (Fig 3D). However, the paternal contribution estimated based on the SNP frequency does not explain the full magnitude of up-regulation in most cases. In addition to paternally contributed transcripts, readenylation of maternal mRNAs can contribute to the observed fold change.

It is intriguing to speculate about functional roles of these sperm-derived RNAs in the zygote. Many of the identified sperm-derived mRNAs in the zygote were significantly enriched in embryonic lethal or sterile phenotypes in RNAi screens. We provided some indications for possible functionality of paternal transcripts by showing that specifically cross-linking paternal RNA in the embryo leads to early embryonic lethality. However, these experiments can only reveal potential functionality of the complement of paternally provided RNAs, and we cannot exclude that the observed lethality is caused by non-incorporated nucleotides cross-linked to proteins or by dysfunction of RNA-binding proteins cross-linked to paternal RNA.

Caenorhabditis elegans sperm is small compared to the oocyte, and it is thought to contribute < 1% of the oocyte volume to the zygote (Browning & Strome, 1996). It is thus conceivable that the transport of mRNAs, from which a large number of proteins can be translated, is a more efficient way for sperm to direct embryogenesis compared to transporting a limited amount of proteins. One speculation could be that sperm delivers mRNAs coding for enzymes that are missing in a cascade or enzymatic cycle in the oocyte, which is thus metabolically arrested until fertilization. The transfer of missing components by sperm into the oocyte could thus kick-start the metabolism of the zygote. Indeed, we find metabolic enzymes in the datasets, but they still require careful independent validation. Moreover, it has been shown that paternal dietary restriction can affect offspring metabolism in worms (Miersch & Döring, 2012) and rodents (Anderson *et al*, 2006; Ng *et al*, 2010) and longevity can be inherited to offspring in worms (Greer *et al*, 2011), however the mode of this trans-generational inheritance is still unknown. It was recently postulated that paternally derived mRNAs or small RNAs could act as a trans-generational carrier of environmental information in rodents (Carone *et al*, 2010; Gapp *et al*, 2014) and/or act as a trans-generational memory of gene expression of preceding generations as suggested recently in worms (Conine *et al*, 2013; Seth *et al*, 2013). Indeed, we found genes associated with stress response among those that were not reproduced in our two independent crossing experiments arguing for a sensitive stress response of males.

Taken together, this study presents, to our knowledge, the first direct evidence that a fertilized metazoan embryo contains hundreds of paternally derived mRNAs and a large proportion of small

non-coding RNAs. Future studies will have to elucidate the functional role of this perhaps unexpected paternal contribution.

Materials and Methods

Caenorhabditis elegans strains and maintenance

Strains were maintained using previously described methods (Brenner, 1974; Stiernagle, 2006) on OP50 seeded NGM plates at permissive temperatures. For all experiments, strains were cultivated at 24°C. Unless otherwise noted, the wild-type strain used was the Bristol N2. The wild-type Hawaiian strain used was CB4856. Feminized strain used was *fem-1*(*hc17*) crossed to TX189[OMA-1::GFP]. The strain used for large-scale male isolation was *fog-2*(*q71*) (see below).

Isolation of 1-cell embryos by eFACS

1-cell and 2-cell stage embryos were obtained by fluorescence-activated cell sorting (eFACS) as described previously (Stoeckius *et al*, 2009) in an FACSAriaIII flow cytometer (BD Biosciences, USA). Microscopic examination of the sorted embryos indicated that the 1-cell embryo sample was virtually pure (> 98% 1-cell stage embryos). Moreover, purity of the stages was further validated by checking for marker gene expression (see below) (Supplementary Fig S1).

Oocyte isolation

Adult wild-type hermaphrodites (N2) were cut using a razorblade in PBS containing 0.5% BSA and 0.02% Tween. Oocytes were picked by mouth pipetting under a stereo microscope (Leica), washed thoroughly in PBS and lyzed in TriZol LS (Invitrogen, USA). Only preparations containing > 98% pure oocytes were used. A fraction of the isolated oocytes was checked for endomitosis by fluorescence microscopy (Zeiss, Germany) with a nuclear dye.

Sperm isolation

Sperm was isolated in principle as described previously (L'Hernault & Roberts, 1995) from male worms obtained from a *fog-2*(*q71*) mutant background strain. Males were cut in cold PBS containing protease inhibitors (Sigma-Aldrich, Germany). Sperm was subsequently purified by filtration (3 × 40 μm, 2 × 10 μm, 1 × 6 μm nylon mesh) and washed twice in cold PBS. Sperm was then activated by incubation in PBS containing 200 μg/ml pronase (Sigma-Aldrich, Germany) for 30 min at 24°C. Sperm purity was checked on an oil immersion microscope and was around 70% spermatids and spermatozoa contaminated with around 30% primary and secondary spermatocytes.

Assessment of sample purity

To validate purity of our 1-cell embryos, we checked our datasets for expression of known zygotic and older embryo marker genes, as well as non-germline tissue-specific transcripts, in our sequencing data. While early embryo and oocyte marker genes are highly expressed (e.g., *oma-1*; Supplementary Fig S1), we did not observe zygotic transcripts that have been described to be expressed in four-to-eight-cell embryos (e.g., *med-1*, *end-3*, *end-1*; Supplementary Fig S1) and did not detect transcripts that are highly expressed in muscle (*myo-3*, *myo-2*, *hlh-1*), neurons (*unc-8, unc-25*), gut (*elt-2, ges-1*) and sperm (*msp-10*, *msp-81*, *msp-56, spe-9*; Supplementary Fig S1). In conclusion, our samples obtained contain 1-cell embryos with high purity.

RNA extraction

RNA was isolated by bead homogenization in a Precellys24 (Precellys, USA) in Trizol LS reagent (Invitrogen, USA) according to the manufacturers' protocols. RNA was coprecipitated with Glycoblue (Ambion, USA) for 30 min at −80°C. Subsequently, RNA was DNAse treated (RQ1 DNAse, Promega) and was reextracted with acid phenol chloroform (Ambion, USA). RNA concentration was measured by means of absorption spectrometry at a wavelength of 260 nm in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA integrity was determined by capillary gel electrophoresis on a Bioanalyzer (Agilent, USA).

PolyA RNA isolation

PolyA mRNA was purified from 500 ng of total RNA using the Dynabeads mRNA Purification kit (Invitrogen, USA) according to the manufacturer's protocol. Depletion of rRNAs was validated by capillary gel electrophoresis on a Bioanalyzer (Agilent, USA). PolyA RNA was subsequently processed for sequencing (see below).

Constructing sequencing libraries for transcriptome analysis

PolyA RNA or RiboMinus RNA was fragmented into approximately 250 nt fragments by chemical fragmentation (200 mM Tris acetate pH 8.2, 500 mM potassium acetate, 150 mM magnesium acetate) at 94°C for exactly 3.5 min in a thermocycler. Fragmented RNAs were isolated with RNA Clean beads (Beckman Coulter, USA) according to manufacturer's instructions. Fractionation was checked by capillary electrophoresis in a RNA Pico 6000 chip using the Bioanalyzer (Agilent Technologies, USA). First-strand cDNA synthesis was accomplished using Superscript III Reverse Transcriptase and random hexamers (Invitrogen, USA), followed by second-strand synthesis using DNA Polymerase I and RNaseH (Invitrogen USA). Double-stranded DNA was purified with Agencourt AMPure beads XP (Beckman Coulter, USA), and quality was checked by capillary gel electrophoresis on the Bioanalyzer with the Agilent DNA 1000 kit (Agilent Technologies, USA). dsDNA libraries subsequently processed for sequencing using the Genomic DNA Sample Prep Kit (Illumina, USA) according to the manufacturer's protocol.

Small RNA library preparation for sequencing

One microgram of total RNA per sample was used for small RNA library preparation. First total RNA was treated with RNA 5′ polyphosphatase (Epicentre Biotechnologies, USA) prior to small RNA library preparation to allow for subsequent cloning of multiphosphorylated endo-siRNAs. Small RNA cloning and library

preparation were then performed with the TruSeq Small RNA Sample Preparation kit (Illumina, USA) according to the manufacturer protocol.

Cluster generation and sequencing

Cluster generation as well as sequencing of the prepared libraries was performed on the Illumina cluster station (Illumina, USA) and HiSeq2000 or GAIIx (Illumina, USA) according to the manufacturer's protocols (Illumina, USA).

Gene models

We used revised and extended modENCODE gene models (Gerstein *et al*, 2010), comprising 64,826 transcripts that correspond to 21,774 different genes.

Processing of mRNA sequencing output

All libraries were sequenced by paired-end sequencing (2 × 101-nt reads) yielding around 60–80 million reads per experiment if performed on GAIIx and more than 100 million reads if performed on HiSeq2000. The paired-end reads of all samples were mapped to the transcriptome sequences using the read alignment software BWA (Li & Durbin, 2010). Prior to read mapping, we removed consecutive strings of base calls with lowest Phred quality score from the 3′ end of the reads and kept only those with a minimum remaining length of 30 bases after trimming. We ran BWA with a minimum seed length of 30 and default parameters otherwise. The fraction of reads mapping to the transcriptome ranged from approximately 60 to 85% for polyA-extraction sequencing. To quantify expression of a given gene locus, we aggregated reads across all isoforms derived from this locus. Reads mapping to multiple loci were distributed uniformly among these loci. Expression was quantified in reads per kilobase of transcript sequence per million mapped reads (RPKM) (Pepke *et al*, 2009), normalizing by the total length of exonic sequence obtained after merging all isoforms. The mean expression μ_i of gene i was computed as the average across biological replicates, and expression variability was estimated by the standard deviation σ_i.

We assigned a confidence value to the expression quantification of gene i, given by max $(0.1, \mu_i/\sigma_i)$. Similarly, we calculated mean and standard deviation of the log2-fold changes between two samples by Gaussian error propagation. We assume a Gaussian distribution of log2-fold changes at each transition and consider the mean as an estimator for the expected log2-fold change. This allows us to infer a z-score and hence a P-value for each log2-fold change.

The reproducibility of transcript fold changes across independent biological replicates was good (Spearman's correlation coefficient > 0.89, Supplementary Fig S1B).

For further analysis, we discarded transcripts with < 2 RPKM.

Mapping and processing of small RNA data

Reads from all small RNA sequencing libraries were mapped to the *C. elegans* genome (WS190). Prior to read mapping, we removed consecutive strings of basecalls with lowest Phred quality score from the 3′ end of the reads and kept only those with a minimum remaining length of 15 bases after trimming. We ran BWA with a minimum seed length of 15 and default parameters otherwise. We assigned a functional annotation to the locus of each read using a hierarchy based on expression of different classes of non-coding RNA. Overlap with annotations of coding and non-coding RNAs in sense and antisense direction was tested in the following order: mature miRNA, miRNA precursor, rRNA, tRNA, snRNA, snoRNA, 21U-RNA, coding exon, coding intron, repeat sequence. If an overlap of at least 5 bases was observed, the read was assigned to the respective class of non-coding RNA and deeper levels of the hierarchy were not tested. We kept only small RNAs with a minimum expression of 2 reads per million (RPM).

For normalization of small RNA expression, we assumed constant abundance of total microRNA in oocytes and embryos. This assumption was validated by RT-qPCR-based expression quantification of 10 different microRNAs for equal numbers of oocytes and embryos normalized to equal concentration of spiked-in external RNA (Supplementary Fig S3B). Within each class of small RNA, we converted expression into reads per one million of microRNA reads. For the inference of differentially regulated small RNAs within each class, we eliminated global changes by computing a linear regression and eliminating the intercept.

Specific classes of small non-coding RNAs antisense to protein-coding genes were extracted based on the length of the mapped reads and the 5′-most nucleotide. The most ubiquitous class, 22G-endo-siRNAs, contains small RNAs of length 22 starting with a G.

Hawaiian cross experiment

To systematically identify the paternal or maternal origin of RNAs in the 1-cell stage embryo, we crossed males from a Hawaiian wild-type isolate (CB4856), isolated by filtration as described previously (L'Hernault & Roberts, 1995), to a feminized hermaphrodite (*fem-1* (*hc17TS*) mutant strain) expressing an OMA-1::GFP fusion protein (referred to as reference strain), which allows for specifically sorting 1-cell embryos and subsequent RNA sequencing of the 1-cell embryo.

Identification of Hawaiian strain-specific mutations

To improve the sensitivity of SNP annotation (or strain-specific RNA editing), we conducted high depth mRNA-seq and rmRNA-seq experiments on the HiSeq2000 platform for whole worms of the Hawaiian strain and corresponding sequencing runs for the reference strain (*fem-1*(*hc17*ts); OMA-1::GFP) used in our study. We sequenced two independent biological replicates, one of those in technical replicates to increase sequencing depth. We then determined Hawaiian-specific nucleotide changes. To this end, we extracted all transcript positions covered by a minimum of five reads in the Hawaiian and one read in the reference strain. To account for sequencing errors, we considered transcript positions as Hawaiian-specific mutations if at most 20% of the reference strain reads but at least 70% of the Hawaiian reads yielded a nucleotide different from the reference genome. A similar procedure was applied to annotate SNPs in small non-coding RNAs based on whole worm and embryo libraries sequenced on the HiSeq2000 platform (Illumina, USA).

For mRNAs, we obtained 16,821 mutations mapping to 5,926 genes, and 3,857 (65%) of those had sufficient read coverage at the SNP position and were expressed in the zygote. Hence, we were able to determine the origin of these zygotic transcripts by looking for SNPs in the embryo transcriptome. We independently determined zygotic SNPs for two biological replicates. Requiring a minimal coverage of six reads and a minimal SNP frequency of 10%, we obtained 1,425 and 955 SNPs, respectively, and a reproducible consensus set of 655 SNPs in 365 transcripts in both replicates. After applying our expression cutoff of 2 RPKM in the oocyte or 1-cell embryo, 192 transcripts have a paternal contribution in both replicates and 164 transcripts have a paternal contribution based on the same SNP position in both replicates.

For small non-coding RNAs, we were able to identify thousands of Hawaiian-specific mutations with the vast majority residing in small RNAs antisense to coding exons. A considerable fraction of these small RNA species was also expressed in offspring 1-cell stage embryos. For a varying fraction of those, depending on the nature of the small RNA, we identified a paternal contribution based on the observed SNP frequency in the embryo.

Hand-picking embryos for validations of miRNA fold changes and Sanger sequencing

Adult wild-type hermaphrodites (N2) were cut using a razorblade in PBS containing 0.5% BSA and 0.02% Tween. 300 one-cell stage embryos and 300 oocytes were collected from cut gravid wild-type (N2) hermaphrodites by mouth pipetting under a stereomicroscope (Leica, Germany). Oocytes and embryos were washed thoroughly in PBS containing 0.5% BSA and 0.02% Tween and lyzed in Trizol LS Reagent (Invitrogen, USA). 0.5 μg of HeLa total RNA was added to each sample to normalize the subsequent RT-qPCR reactions on an exogenous spike.

Validation of observed miRNA expression patterns by TaqMan miRNA qPCR assays

The fold changes that were computed for miRNAs from the deep sequencing data between oocytes and 1-cell stage embryos were validated by TaqMan miRNA qRT-PCR assays (Applied Biosystems) on independently handpicked oocytes and 1-cell stage embryos. MicroRNA TaqMan PCR assays were performed following the recommendations of the manufacturer (Applied Biosystems). A TaqMan assay for human miR-34 was used as a normalization standard (equal amounts of human RNA were spiked prior to RNA extraction).

^{3}HU, 6SG, and 4SU labeling and crossing experiments

Worms were grown for one generation on NGM plates containing the labeled nucleotide analogues (^{3}HU; 4SU; or 6SG) at 2 mM final concentration as described previously (Jungkamp *et al*, 2011). We extensively tested incorporation of these nucleotides into the RNA of the nematode for other projects that are currently ongoing in the laboratory. Adult males were isolated by filtration as described previously (L'Hernault & Roberts, 1995). Only male preparations with >98% purity determined under a stereomicroscope were used for subsequent crosses. Labeled (or non-labeled) males were crossed with non-labeled (or labeled) feminized hermaphrodites (*fem-1*(*hc17*) mutants) grown at restrictive temperature. After 3 h, embryos were isolated from gravid worms by bleaching (Stiernagle, 2006) for testing RNA labeling (see below) or worms were irradiated for viability assays (see below).

Dot blot assays to measure biotinylated RNA

To detect and quantify 6SG incorporation into RNA, RNA was thiol-specifically biotinylated and analyzed on a dot blot as described previously (Doelken *et al*, 2008). Typically, 10 μg of total biotinylated RNA was spotted onto a membrane for dot blot analysis. The dot blot image was then digitalized using the Fuji LAS4000 and quantified using ImageJ.

Scintillation counting of ^{3}H-labeled RNA

^{3}HU-labeled RNA was measured in two dilutions in a scintillation counter according to manufacturer (Beckmann, USA).

Cross-linking of 4SU- and 6SG-labeled worms to assay embryonic viability

For viability assays, embryos of labeled or non-labeled worms were irradiated *in utero* with 3 J UV 365 nm light as described previously (Jungkamp *et al*, 2011). Embryos were subsequently extracted by cutting and picked at the desired stage by hand pipetting. Embryos were allowed to hatch in M9 at 20°C.

GO term analysis

GO term analysis was performed in R using the GOstats package. Over-represented GO terms were computed against the background of all genes expressed (> 2 RPKM) in oocytes or embryos.

RNAi phenotype enrichment analysis

For the enrichment analysis of RNAi phenotypes, data for all available RNAi experiments (WS190) were downloaded from Wormbase.org. All genes with support from less than two experiments were discarded. Phenotypes were considered if observed in at least 50% of all experiments performed on the gene of interest.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

MS and NR devised the project. MS designed and performed all experiments. DG designed and performed the entire computational analyses. NR supervised MS and DG. MS, DG, and NR interpreted the data. MS, DG and NR wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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